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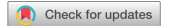
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ORIGINAL RESEARCH



## Molecular and clinical characterization of TIM-3 in glioma through 1,024 samples

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### ABSTRACT

**Background:** Researches on immunotherapy of glioma has been increasing exponentially in recent years. However, autoimmune-like side effects of current immune checkpoint blockade hindered the clinical application of immunotherapy in glioma. The discovery of the TIM-3, a tumor-specific immune checkpoint, has shed a new light on solution of this dilemma. We aimed at investigating the role of TIM-3 at transcriptome level and its relationship with clinical practice in glioma.

**Methods:** A cohort of 325 glioma patients with RNA-seq data from Chinese Glioma Genome Atlas (CGGA project) was analyzed, and the results were well validated in TCGA RNA-seq data of 699 gliomas. R language was used as the main tool for statistical analysis and graphical work.

**Results:** TIM-3 was enriched in glioblastoma (the most malignant glioma) and IDH-wildtype glioma. TIM-3 can act as a potential marker for mesenchymal molecular subtype according to TCGA transcriptional classification scheme in glioma. TIM-3 was closely related to immune functions in glioma, especially T cell mediated immune response to tumor cell and T cell mediated cytotoxicity directed against tumor cell target. Moreover, TIM-3 and PD-L1 played almost exactly the same inflammatory activation functions in glioma. Clinically, high expression of TIM-3 was an independent indicator of poor prognosis.

**Conclusion:** The expression of TIM-3 is closely related to the pathology and molecular pathology of glioma. Meanwhile, in glioma TIM-3 plays a specific role in T cell tumor immune response. Therefore, TIM-3 is a promising target for immunotherapeutic strategies, providing an alternative treatment when glioma gains resistance to antibodies of PD-1/PD-L1.

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Glioma; immune response; inflammatory activation; prognosis; TIM-3





## Introduction


Glioma represents the most common and malignant brain tumor in adults, characterized by a high recurrence rate and high fatality rate.<sup>1,2</sup> Despite multimodal conventional therapy, including neurosurgical resection and radiotherapy with concomitant and adjuvant alkylating agent temozolomide chemotherapy, glioma remains a leading cause of death in human cancer. Within decades, amounts of researches on molecular markers and molecular targeted drugs produced very limited effect in prolonging life expectancies of glioma patients. The discovery of intracranial lymphatic system has brought a new theoretical basis and new hope for brain tumor immunotherapy.<sup>3</sup>

Driven by the success of immune checkpoint blockade in other cancers, researches on immunotherapy of glioma has increased exponentially in the past few years.<sup>4,5</sup> However, most glioma have been proved to be refractory to current immunotherapies. This has raised our interest in finding novel immune checkpoints directly targeting tumor cells in glioma.

T cell immunoglobulin domain and mucin domain 3 (TIM3) is a membrane protein selectively expressed on interferon-gamma-secreting CD4<sup>+</sup> T helper 1 (Th1) and CD8<sup>+</sup> T cytotoxic (Tc1) cells.<sup>6</sup> Emerging data suggest that Tim-3 takes a center stage in T cell exhaustion by triggering cell death: T cells fail to exert effective functions such as cytotoxicity and cytokine secretion in response to antigen and tumor cell stimulation.<sup>7,8</sup> TIM-3, as a key immune checkpoint in tumor-induced immune suppression, exhibits several unique features that make it an intriguing candidate for the next wave of therapies in cancer.<sup>9</sup>

As a novel immune checkpoint, TIM-3 is gaining more and more attention in both solid and hematologic malignancies.<sup>10</sup> However, we reviewed the current evidence and failed to find a single comprehensive report about TIM-3 in glioma. Taking advantage of Chinese Glioma Genome Atlas (CGGA) data set, we gathered RNA-seq data of 325 glioma samples to take an integrative investigation of TIM-3 in glioma. Moreover, our

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findings were well validated in another RNA-seq data set of 699 gliomas obtained from TCGA network (<http://cancergenome.nih.gov>). This is the first integrative study characterizing TIM-3 expression in whole grade glioma molecularly and clinically. And we believe that TIM-3 will be a hot ticket in glioma immunotherapy.

## Patients and methods

### Patients and samples

This study was approved by the Beijing Tiantan Hospital institutional review board (IRB), and written informed consent was obtained from each patient. Only samples with greater than 80% tumor cells were selected. Transcriptome sequencing data of glioma samples were from CGGA generated with Illumina HiSeq platform. Overall survival was estimated from the date of diagnosis to the date of either death or last follow-up. Methods to detect IDH mutation state has been described in our previous study.<sup>11</sup> The Cancer Genome Atlas (TCGA) mRNA-seq database was downloaded from public databases (<https://tcga-data.nci.nih.gov/tcga/tcgaDownload.jsp>). The primary GSC (glioma stem-like cells) gene expression array data set was downloaded from GSE67089.

### Gene set variation analysis (GSVA) analysis

After Spearman correlation analysis, gene ontology (GO) analysis of the most correlated genes was constructed by Heatmap. The GO geneset was downloaded from AmiGO 2 Web portal (<http://amigo.geneontology.org/amigo/landing>). Inflammatory-related metagenes has been described in our previous study.<sup>5</sup>

### Statistical analysis

The prognostic value of TIM-3 was estimated by Kaplan–Meier analysis and Cox proportional hazard model analysis using SPSS statistical software (version 19). Other statistical computations and figures drawing were performed with several packages (ggplot2, pheatmap, pROC and corrgram) in the statistical software environment R, version 3.3.2 (<http://www.r-project.org>). For all statistical methods,  $p < 0.05$  was considered as significant difference.

## Results

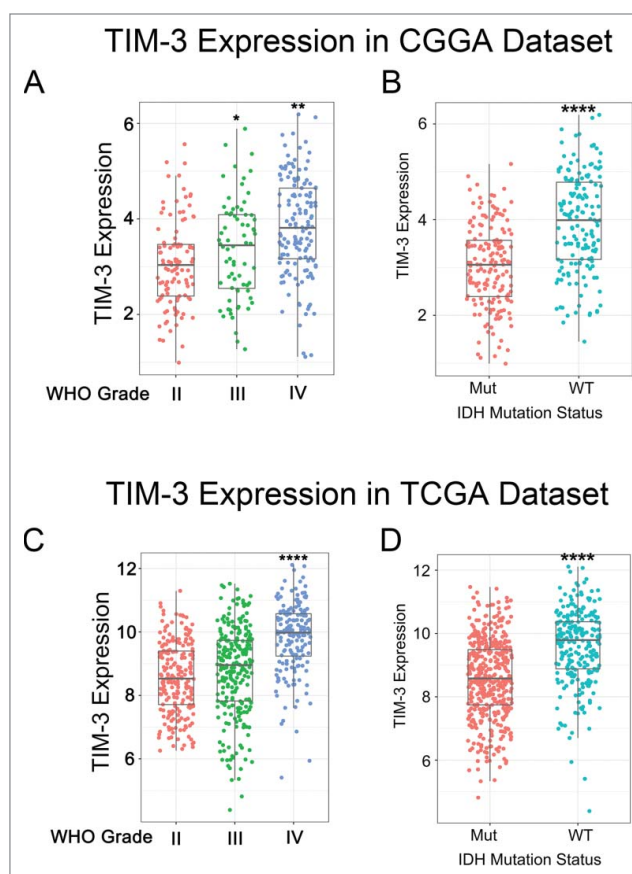
### TIM-3 was enriched in glioblastoma and IDH-wildtype glioma

To characterize the expression pattern of TIM-3 in glioma, we examined the RNA-sequencing data of glioma from CGGA and TCGA database. Compared to WHO grade II and grade III glioma, glioblastoma (WHO IV) showed the highest TIM-3 expression in CGGA database (Fig. 1A). This result was well validated in TCGA RNA-seq data (Fig. 1C). These results suggested that high expression of TIM-3 was a sign of high malignancy of glioma, in consistency with other malignant tumors reported previously.<sup>12–14</sup> It is acknowledged that IDH mutation plays a very important role in the development and progression of glioma.<sup>15,16</sup> Consequently, we also explored the relationship

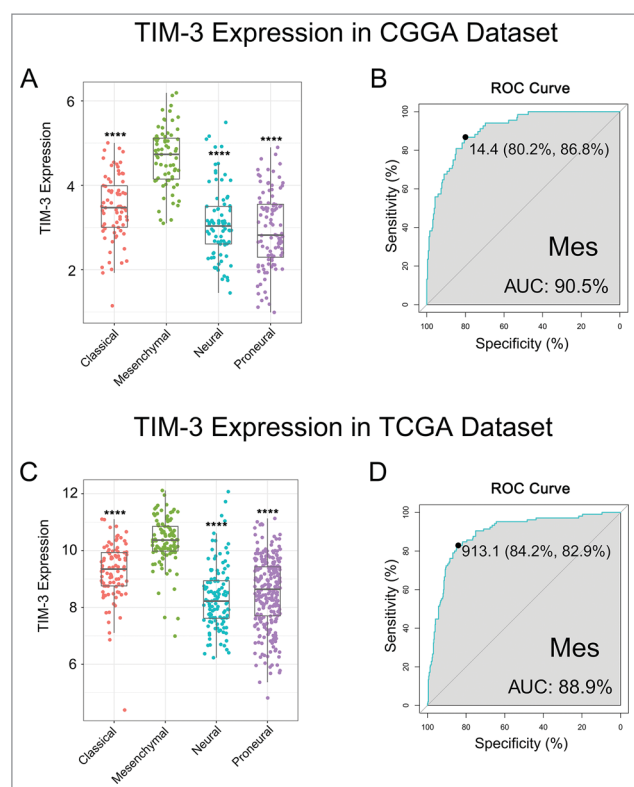
between TIM-3 expression level and IDH mutation status and found that TIM-3 was highly enriched in IDH wildtype glioma in both CGGA and TCGA data set (Figs. 1B and D and Fig. S1). This finding indicated that TIM-3 check point related immune responses were more prevalent in IDH wildtype glioma. Moreover, we found that TIM-3 was specifically expressed in GSC cells (Fig. S2), which was also found in acute myeloid leukemia.<sup>17</sup>

### TIM-3 was a potential marker for mesenchymal molecular subtype glioma

To find out the molecular expression pattern of TIM-3, we asked the distribution of TIM-3 in different molecular subtypes defined by TCGA network.<sup>18</sup> When compared with other three subtypes respectively, TIM-3 was significantly upregulated in mesenchymal subtype in CGGA cohort as well as in TCGA cohort (Figs. 2A and C). To further validate this finding, ROC curves for TIM-3 expression and mesenchymal subtype of all grade glioma were performed. Surprisingly, area under the curve (AUC) were up to 90.5% and 88.9% in CGGA and TCGA data set, respectively (Figs. 2B and D). These results suggested that TIM-3 was highly specifically expressed in mesenchymal subtype glioma. Based on these findings, we inferred that TIM-3 may play important biologic functions in glioma. To validate our hypothesis, biologic function analyses need to be performed.



**Figure 1.** TIM-3 was significantly upregulated in glioblastoma and IDH-wildtype glioma. (A, C) TIM-3 was significantly increased in glioblastoma (WHO IV) in CGGA and TCGA data set. (B, D) TIM-3 was significantly increased in IDH-wildtype gliomas in CGGA and TCGA data set. \*, \*\* and \*\*\*\* indicate  $p < 0.05$ ,  $p < 0.01$  and  $p < 0.0001$ , respectively.



**Figure 2.** TIM-3 was highly enriched in mesenchymal molecular subtype glioma. (A, C) TIM-3 expression pattern in different molecular subtypes in CGGA and TCGA data set. (B, D) ROC curve analysis showed that TIM-3 had highly sensitivity and specificity to predict mesenchymal subtype in CGGA and TCGA database. \*\*\*\* indicates  $p < 0.0001$ .

### **TIM-3 was closely related to immune functions in glioma**

To clarify the biologic role of TIM-3 in glioma, we performed GO analysis. First, we created a gene list that strongly correlated with TIM-3 by Pearson correlation analysis (Pearson  $|R| > 0.6$ ). Finally, there were 390 genes in CGGA gene list and 495 genes in TCGA gene list. Then, we explored the biofunction of these genes respectively by GO analysis in DAVID Bioinformatics Resources 6.8. When the gene function was sorted by  $p$  value in increasing order, genes most relevant to TIM-3 were mostly involved in immune response and inflammatory response in both CGGA and TCGA database (Figs. 3A and B). To further explore the immune function of TIM-3 in specific immune functions, we then studied the role of the specific immune roles of TIM-3 in immune response and inflammatory activities.

### **TIM-3 related immune response**

To further explore the role of TIM-3 in the immune response in glioma, we found out certain genesets related to the immune response from the AmiGO 2 Web portal. When removed insignificant genes (gene expression was 0 more than half patients), the remaining 1,540 genes were eligible for subsequent analysis. We selected the genes that were most relevant to TIM-3 (Pearson  $|R| > 0.4$ ) for the heatmap drawing. Among the 411 selected-genes, 397 genes were significantly positively correlated with TIM-3 expression and 14 genes were significantly negatively correlated with Tim-3 expression (Figs. 3C and D).

A detailed list of these genes was shown in Table S1. Above analysis found that TIM-3 was positively correlated with most immune responses and negatively correlated with a small number of immune responses in glioma. A thorough study of this result may reveal the important role of TIM-3 in gliomas.

### **The relationship between TIM-3 and T cell immunity**

TIM-3 promotes tumor progression in a variety of tumors by inhibiting T cell immune function.<sup>19,20</sup> Whether it has the same functions in gliomas remain an enigma. To elucidate the relationship between TIM-3 and T cell immune in glioma, GSVA analysis was performed. As shown in Figs. 4A and B, TIM-3 was positively correlated with T-helper 1/2 type immune response, T-helper 1/2 cell cytokine production and natural killer cell mediated cytotoxicity directed against tumor cell target. Meanwhile, it was negatively correlated with T cell mediated immune response to tumor cell and T cell mediated cytotoxicity directed against tumor cell target. Importantly, this result can be verified mutually in CGGA and TCGA databases. This also confirmed that TIM-3 played an inhibitory role in T cell immune to tumor in glioma. The special immune function of TIM-3 revealed the mechanism of this gene in glioma.

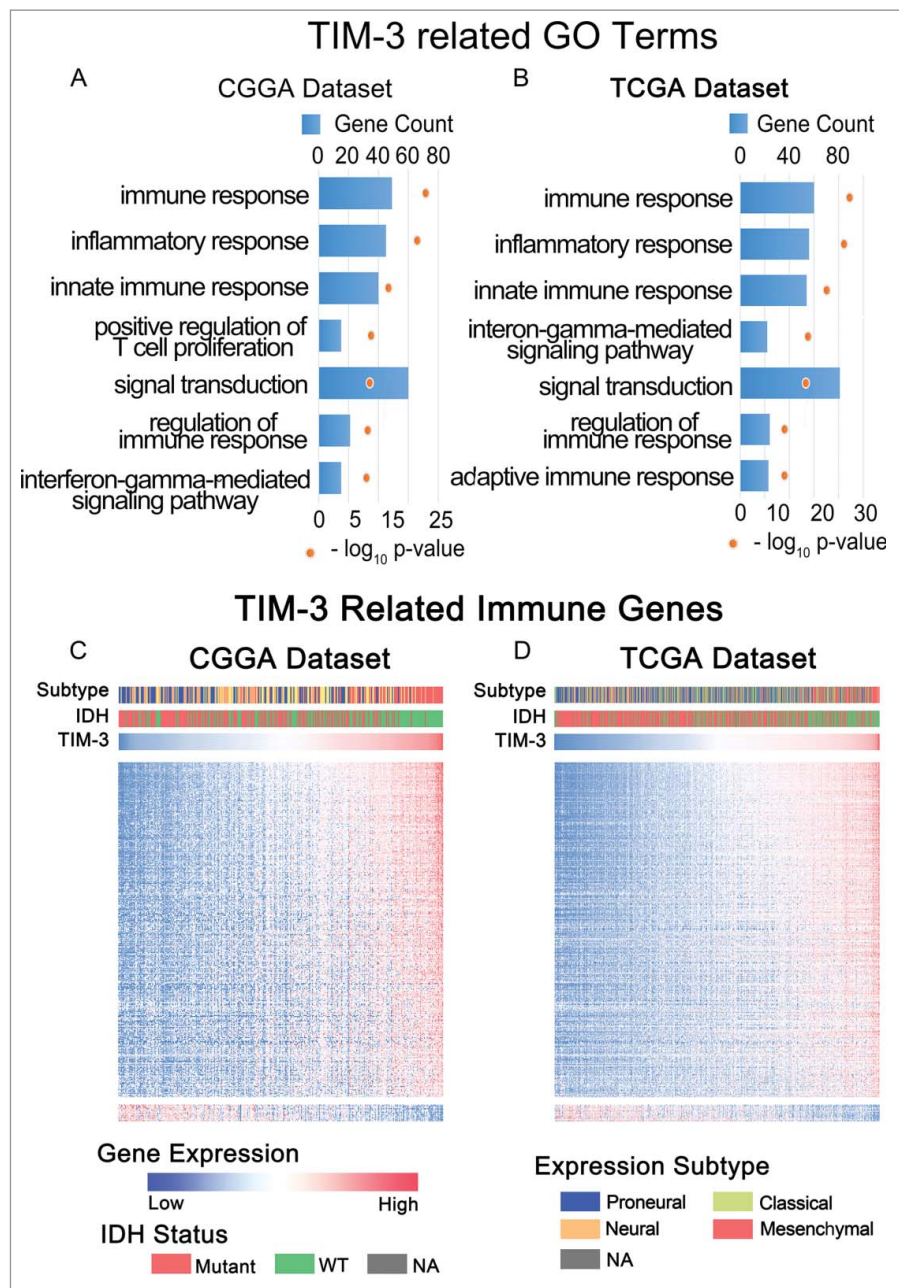
### **The relationship between TIM-3 and inflammatory activities**

As revealed above, TIM-3 also played an important role in the inflammatory response in glioma. We used the method described previously to specifically analyze the role of TIM-3 in the glioma inflammatory response.<sup>5</sup> To our surprise, TIM-3 showed exactly similar pattern to inflammation activities as PD-L1 we reported earlier<sup>5</sup> (Figs. 4C and D). Both genes were positively associated with HCK, LCK and MHC-I, but were negatively associated with IgG. These results suggested that TIM-3 and PD-L1 play almost exactly the same inflammatory activation functions (they were macrophages and T cells signaling transduction suppressors, but not much involved in B lineage related immune responses) in glioma. This finding was consistent with the above results and further confirmed the important immune function of TIM-3 in glioma.

### **TIM-3 predicted worse survival in glioma**

As TIM-3 showed robust negative relationship with T cell tumor immune response, we additional analysis the prognostic value of TIM-3. We analyzed the prognosis of a total of 1,024 glioma patients by Kaplan–Meier method. Similar with most malignancies, overexpression of TIM-3 predicted significantly poor overall survival in both CGGA and TCGA databases (Figs. 5A and B). Furthermore, Cox regression analysis was performed additionally, verifying the independence of the clinical prognostic significance of TIM-3 in glioma. In the above two databases, it was showed that TIM-3, Age at Diagnosis, WHO Grade, KPS Score and IDH Status were significantly associated with overall survival. On multivariate analysis, the expression of TIM-3 was also a significant factor after adjusting for the clinical factors mentioned above (Table 1 and Table S2). These findings indicated that TIM-3 predicted poor prognosis in





**Figure 3.** TIM-3 was closely related to immune functions in glioma. (A, B) Gene ontology analysis showed that TIM-3 was mostly involved in immune response and inflammatory response in CGGA and TCGA database. (C, D) Most immune response related genes were significantly positively correlated with TIM-3 expression, while there were still a small number of genes were significantly negatively correlated with Tim-3 expression.

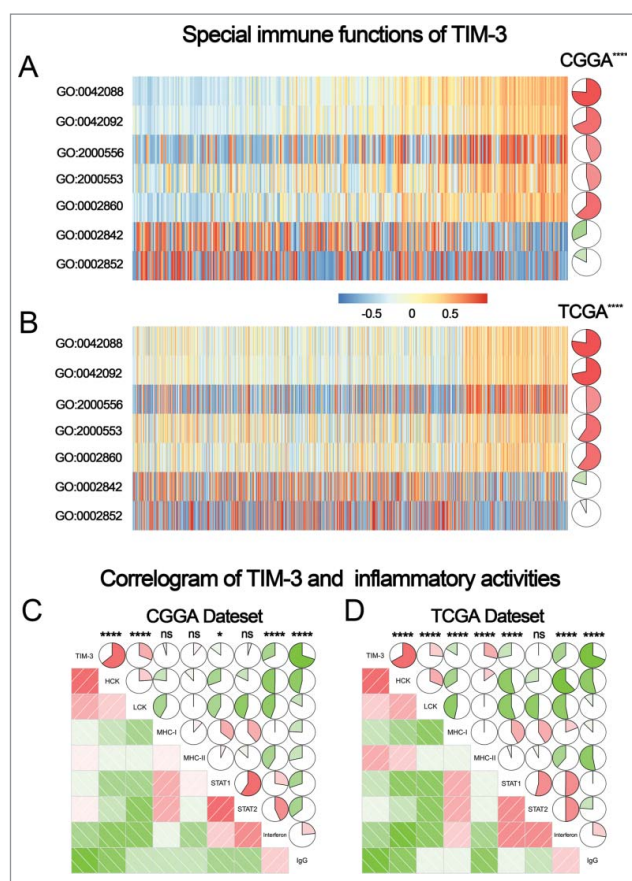
glioma due to suppressive effect on T-cell-related immune response, especially T cell immune to tumor.

## Discussion

Glioma is one of the highly fatal diseases that affects human health severely. Aside from temozolomide, for decades, no major breakthrough was achieved to improve the prognosis of glioma patients.<sup>2,21</sup> Therefore, new therapeutic approaches are urgently needed. In recent years, as a novel therapeutic approach of glioma patients, immunotherapy has shown a promising prospect.<sup>22</sup> At present, researches on the field of neural tumor immunotherapy are mainly focus on CTLA-4 and PD-1/PD-L1 blockade.<sup>23,24</sup> However, since the

autoimmune-like side effects of current immune checkpoint blockade, immunotherapy is difficult to be widely applied. This is the so-called “Cancer Immunology at the Crossroads.”<sup>10</sup> Fortunately, at this critical moment, people discovered a novel immune checkpoint receptor-TIM-3. This novel immune checkpoint exhibits several unique advantages that make it an intriguing candidate for immunotherapy of glioma in the future.

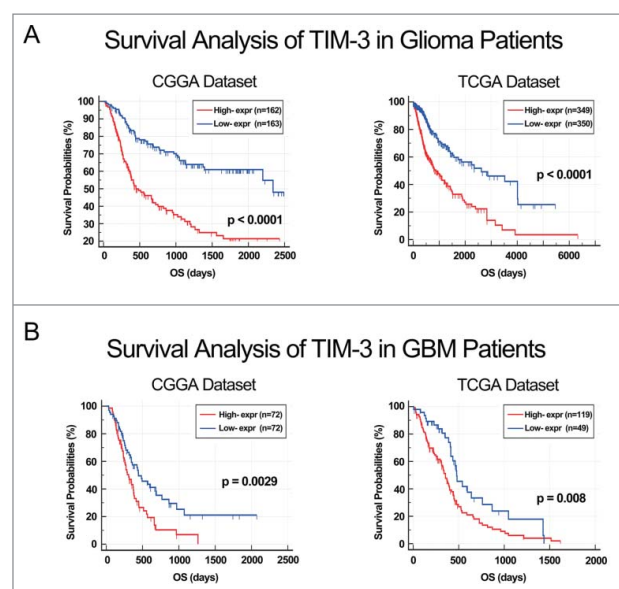
Here, we analyzed the TIM-3 expression in the RNA-seq data of 1,024 glioma patients. As expected, TIM-3 expression was significantly upregulated in higher malignant pathological type gliomas. Moreover, we also found that high expression of TIM-3 was highly enriched in the phenotype of known malignant molecules, such as IDH wildtype state, mesenchymal



**Figure 4.** TIM-3-related T cell immunity and inflammatory activities in glioma. (A, B) The relationship between TIM-3 and T cell immunity in CGGA and TCGA data set. (C, D) The relationship between TIM-3 and inflammatory activities in glioma. The correlation between TIM-3 and other functions was analyzed by Pearson correlation analysis. ns, \* and \*\*\*\* indicate no statistical difference,  $p < 0.05$  and  $p < 0.0001$ , respectively. GO:0042088: T-helper 1 type immune response, GO:0042092: T-helper 2 type immune response, GO:2000556: positive regulation of T-helper 1 cell cytokine production, GO:2000553: positive regulation of T-helper 2 cell cytokine production, GO:0002860: positive regulation of natural killer cell mediated cytotoxicity directed against tumor cell target, GO:0002842: positive regulation of T cell mediated immune response to tumor cell, GO:0002852: regulation of T cell mediated cytotoxicity directed against tumor cell target.

phenotype and glioma stem cells. All these results indicated that TIM-3 expression was associated with more malignant biologic process as other solid and hematologic malignancies.<sup>25,26</sup> It is most likely that these malignant biologic behaviors have contributed to tumor recurrence and therapy resistance. Revealing mechanism of TIM-3 in glioma may be the key to triumphing over this deadly disease.

Through an in-depth analysis of the biologic functions of TIM-3 in glioma, we found that TIM-3 played an important role in the glioma immune response, especially in tumor-induced immune suppression. This special effect of TIM-3 has also been observed in other tumors.<sup>7,8,20</sup> This may be accused to the unique presence of TIM-3 Tregs in tumor tissue, which was a unique advantage over other immune checkpoints.<sup>27-29</sup> Furthermore, another unique advantage of TIM3 was that intracellular tail of TIM-3 did not contain any immunoreceptor tyrosine-based inhibition motifs (ITIM) or immunoreceptor tyrosine-based switch motifs (ITSM).<sup>30</sup> These advantages effectively avoided the major deficiencies of current immunotherapy



**Figure 5.** ALDH1A3 was a prognostic factor in glioma and GBM patients. (A) Kaplan–Meier survival analysis showed that high expression of TIM-3 conferred a worse prognosis in glioma patients. (B) Kaplan–Meier survival analysis showed that high expression of TIM-3 conferred a worse prognosis in GBM patients.

and highlighted its value in glioma immunotherapy. As an immune inhibitory receptor, suppressing the inflammatory response and autoimmunity are the natural property of TIM-3.<sup>31,32</sup> We found that TIM-3 played almost exactly the same role in inflammatory response as PD-L1 in glioma. Together, these data indicated that TIM-3 and PD-L1 played a synergistic role in tumor inflammatory response to promote the development of a severe dysfunctional phenotype in T cells in glioma as reported in other cancers.<sup>33,34</sup> Thus, theoretically, antibodies derived to block the stimulation of TIM-3 should have the same effect as PD-L1 suppression. Multiple preclinical and clinical targeting TIM-3 have yielded consistent results with the theory in various tumors, particularly melanoma and non-small-cell lung cancer.<sup>35,36</sup> A research published in the latest issue of *Clin Cancer Res* reported that TIM-3/PD-1 co-blockade was more effective than either TIM-3 or PD-1 blockade alone at restoring inflammation activation and improving survival time from glioma-bearing murine.<sup>37</sup> This gratifying discovery provided a reliable support for our research. Furthermore, TIM-3 will also have a profound impact on studies of glioma

**Table 1.** Univariate and multivariate analysis of clinical prognostic parameters in CGGA Dataset.

Variable	Univariate analysis		Multivariate analysis	
	HR (95% CI)	$p$ value	HR (95% CI)	$p$ value
TIM-3 expression	1.038 (1.027–1.050)	<0.0001	1.023 (1.007–1.040)	0.005
Age at diagnosis	1.038 (1.023–1.054)	<0.0001	0.991 (0.969–1.014)	0.452
WHO grade	3.477 (2.716–4.452)	<0.0001	2.752 (1.961–3.862)	<0.0001
KPS score	0.972 (0.962–0.982)	<0.0001	0.972 (0.960–0.984)	<0.0001
IDH mutation	0.228 (0.158–0.329)	<0.0001	0.763 (0.433–1.344)	0.348

stem cells if it can be a therapeutic target of glioma stem cells. This hypothesis has already been validated in acute myeloid leukemia stem cells.<sup>38</sup> Whether it is the same case in glioma stem cells or not, still need further studies. Collectively, these data strongly support the potential of TIM-3 blockade for the immunotherapy of glioma.

The future of glioma treatment most likely resides in combinatorial approaches, with administration of conventional treatments (surgery, radiochemotherapy) and immunotherapy complement each other. Our works will greatly promote the immunotherapy of glioma into a new era.

## Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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